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Subclassification of β-adrenoceptors responsible for steroidogenesis in primary cultures of bovine adrenocortical zona fasciculata/reticularis cells

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1 Forty eight hour primary cultures of purified bovine adrenocortical zona fasciculata/reticularis cells secreted hydrocortisone in response to stimulation with β-adrenoceptor agonists. The observed order of potency was isoprenaline > noradrenaline > dobutamine > salbutamol > BRL37344.

2 Salbutamol acted as a partial agonist on these cells hence suggesting the presence of a β_1-adrenoceptor.

3 Schild analysis of the hydrocortisone response to isoprenaline showed that the selective β_1-antagonist practolol and the selective β_2-antagonist ICI118,551 gave pA_2 values of 6.85 and 7.17, respectively. These values were in close agreement with corresponding pA_2 values previously obtained for the β_1-adrenoceptor.

4 We conclude that β_1-adrenoceptors are responsible for mediating catecholamine-stimulated hydrocortisone secretion from primary cultures of bovine zona fasciculata/reticularis cells.

Introduction

Adrenocorticotrophic hormone (ACTH) and, in some species, angiotensin II are known regulators of steroidogenesis in the adrenal cortex (Gill *et al.*, 1977; Tait *et al.*, 1980). However, there is also evidence for innervation of the adrenal cortex in man (Mikhail & Amin, 1969), the rat (Kleitman & Holzwarth, 1985) and the mouse (Migally, 1979), and this has been suggested as another means of controlling adrenocortical steroidogenesis and possibly adrenal cell growth.

We have previously shown that primary cultures of bovine zona fasciculata/reticularis (ZFR) cells produce hydrocortisone in response to stimulation with catecholamines, and that this adrenergic stimulation of steroidogenesis is mediated by β-adrenoceptors (Walker *et al.*, 1988). The subclass of β-adrenoceptors which is involved in this response—either β_1 or β_2 as classified by Furchgott (1972), or the more recently characterised β_3 subclass (Arch *et al.*, 1984; Kaumann, 1989)—is unknown.

It is not yet clear in bovine or other species whether adrenergic control of hydrocortisone secretion at the level of the adrenal cortex occurs *in vivo* and, if so, whether this depends on adrenergic innervation, on the effects of circulating catecholamines, or even on catecholamines locally derived from the adrenal medulla. Ungar (1979) suggested that, in general, β_1-adrenoceptors tend to be innervated by adrenergic neurones, whereas β_2-adrenoceptors respond mainly to blood-borne catecholamines. On this basis, determination of the subclass of β-adrenoceptor should provide valuable evidence as to whether adrenergic control of the adrenal cortex is by direct innervation or via circulating catecholamines.

Traditional receptor classification is based on determination of antagonist pA_2 values (Schild, 1947; Kenakin, 1982), and comparison of a range of selective agonists (Lands *et al.*, 1967). Subclassification of β-adrenoceptors has been successfully demonstrated with the selective β_1-antagonist practolol (Dunlop & Shanks, 1968) and the selective β_2-antagonist ICI118,551 (Bilski *et al.*, 1983). These antagonists have been used in this present study. The effects of the selective β-agonists noradrenaline (β_1), salbutamol (β_2), dobutamine (β_1), isoprenaline (β_1/β_2), and BRL37344 (β_3) were also compared.

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Methods

Cell culture and stimulation

Bovine adrenal ZFR cells were prepared as described by Walker *et al.* (1988) using collgenase digestion, and purified by the column-filtration method developed by McDougall *et al.* (1979). This procedure gives a preparation essentially free from zona glomerulosa cell and medullary cell contamination (Williams *et al.*, 1989). The ZFR cells were dispersed into 12-well culture dishes (1.5 cm wells) at 250,000 cells per well in Ham's F10 medium containing 10% (v/v) CPSR5, penicillin ($50\,iu\,ml^{-1}$), streptomycin ($50\,\mu g\,ml^{-1}$) and amphotericin B ($2.5\,\mu g\,ml^{-1}$), and cultured at 37°C under 5% CO_2. After 24 h (day 2), the medium was replaced with 1 ml of identical fresh medium.

Experiments were carried out 48 h after initial plating (day 3). Medium was removed and cells were washed twice with 1 ml of Earl's balanced salt (EBS) solution containing 0.2% bovine serum albumin (BSA) and 0.1% added glucose (EBS/BSA/glucose). Agonists and antagonists were made up in EBS/BSA/glucose and added to the cells, giving a final volume of 1 ml per well. Antagonists were added 1 min before the addition of any agonists. Stimulation was carried out for 1 h under the same incubation conditions as those used to culture the cells and, at the end of this period, the medium overlying the cells was removed and stored at −20°C before assay for hydrocortisone.

Hydrocortisone was measured by radioimmunoassay as described by Gray *et al.* (1983). The inter-assay CV was 10% or less over the working range of the assay.

Statistical analysis

Experiments were carried out on cells from at least 3 separate cell preparations for determination of agonist potencies and for Schild analysis of each antagonist. Within each experiment, triplicate determinations were carried out for each combination of agonist and antagonist.

For the estimation of antagonist pA_2 values, dose-response curves were tested for parallelism by analysis of covariance

using the SPSS-X statistical package produced by Edinburgh University Computing Service. Schild regression lines were fitted with a least-squares fit by a Casio fx-180P programmable calculator and confidence limits calculated.

Materials

The source of all cell culture and radioimmunoassay materials is described in Walker *et al.* (1988). The controlled process serum replacement No. 5 (CPSR5) was obtained from Sigma Chemical Company, Poole, Dorset, U.K.

Noradrenaline was obtained from Winthrop, Guildford, Surrey, U.K.; salbutamol from Allen & Hanburys Ltd., London, U.K.; dobutamine from Eli Lilly & Co. Ltd., Basingstoke, U.K. and isoprenaline from Macarthy Medical, Romford, U.K. BRL37344 ((**R*R***)-(±)-4-[2-[2-hydroxy-2-(3-chlorophenyl)-ethylamino]propyl]phenoxyacetic acid. Na salt) was a generous gift from Beecham Pharmaceuticals, Epsom, Surrey, U.K. Practolol was obtained from ICI plc, Macclesfield, Cheshire, U.K. and ICI 118,551 (erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) was a generous gift from the same company.

Results

The effects of various selective β-agonists on hydrocortisone secretion

The effects of increasing concentrations of isoprenaline, noradrenaline, salbutamol, dobutamine and BRL37344 were tested on cells on day 3 of culture (Figure 1). The agonists had relative potencies as follows: isoprenaline > noradrenaline > dobutamine > salbutamol > BRL37344. Isoprenaline, noradrenaline and dobutamine all produced the same maximum response. Salbutamol and BRL37344 gave approximately 70% and 19% of the maximum response, respectively.

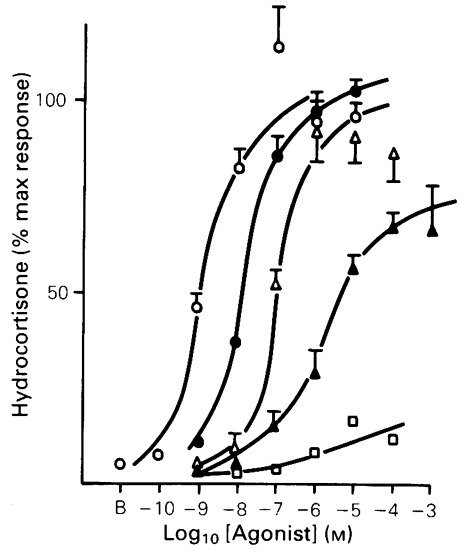

Figure 1 Dose-response curves for the secretion of hydrocortisone produced upon stimulation of purified bovine adrenocortical zona fasciculata/reticularis cells with isoprenaline (○), noradrenaline (●), dobutamine (△), salbutamol (▲) and BRL37344A (□) B = basal cortisol production.

The effects of the antagonists practolol and ICI118,551 on the isoprenaline dose-response curve

A series of dose-response curves for the effect of isoprenaline on hydrocortisone secretion were set up in the presence of increasing doses of either practolol or ICI118,551. Representative experiments for practolol and ICI118,551 are shown in Figures 2 and 3, respectively. The dose-response lines for each concentration of antagonist were judged to be parallel by analysis of covariance. Experiments were repeated on cells from 4 separate cell preparations for practolol and 3 separate cell preparations for ICI118,551.

The dose-ratio (DR) for each concentration of antagonist was obtained, Schild plots of $\log_{10}$ (DR − 1) versus antagonist

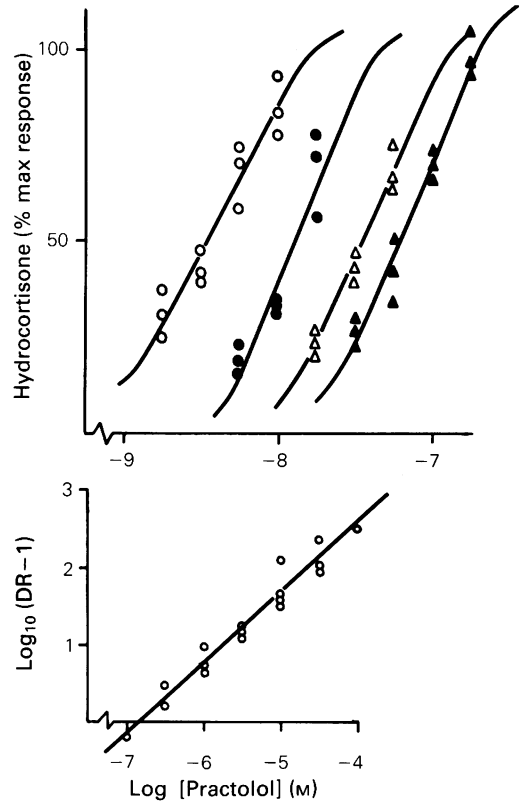

Figure 2 Representative experiment showing dose-response curves for the secretion of hydrocortisone produced on stimulation with isoprenaline, alone (○) and in the presence of increasing concentrations of practolol $10^{-6.5}$ M (●), 10^{-6} M (△), $10^{-5.5}$ M (▲). Inset: Schild regression—least squares fit of $\log_{10}$ (DR − 1) versus $\log_{10}$ [practolol] where DR = dose-ratio. Cumulative data from 4 separate cell preparations.

Table 1 Comparison of experimental and published data for practolol and ICI118,551

Antagonist	*Experimental* pA_2	*95% CL*	*Slope*	*95% CL*	*Published* pA_2
		6.67		0.84	
Practolol	6.85		0.90		6.80[1] (β_1)
		7.06		0.96	
		7.03		1.01	
ICI118,551	7.14		0.99		7.17[2] (β_1)
		7.28		0.97	9.26[2] (β_2)

95% CL = 95% confidence limits. Original references are [1]Kenakin & Black (1978), [2]Bilski *et al.* (1983).

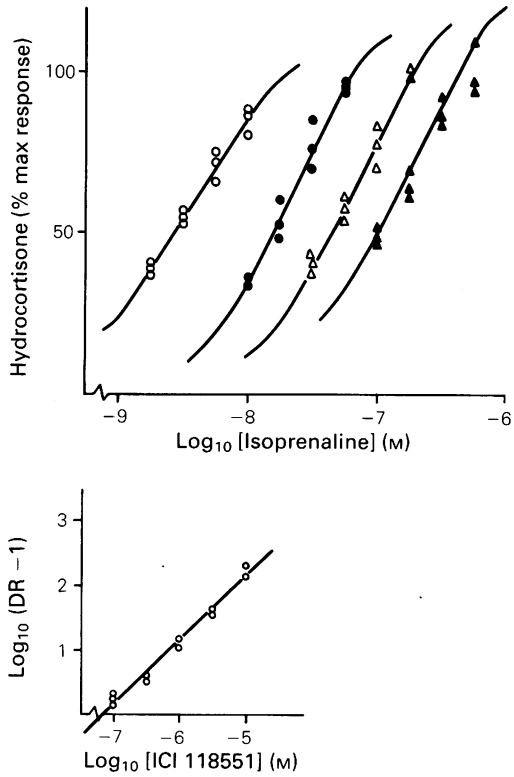

Figure 3 Representative experiment showing dose-response curves for the secretion of hydrocortisone produced on stimulation with isoprenaline, alonr (○) and in the presence of increasing concentrations of ICI118,551 $10^{-6.5}$M (●), 10^{-6}M (△) $10^{-5.5}$M (▲). Inset: Schild regression—least squares fit of $\log_{10}$(DR − 1) versus $\log_{10}$[ICI118,551] where DR = dose-ratio. Cumulative data from 3 separate cell preparations.

concentration plotted (lower sections in Figures 2 and 3) and used to calculate pA_2 values for each antagonist and to obtain 95% confidence limits. The slope of the regression line and 95% confidence limits were estimated similarly. Results for both antagonists are shown in Table 1.

Neither practolol nor ICI118,551 caused hydrocortisone secretion and therefore had no intrinsic agonist effects on the cells.

Discussion

The results establish the existence of β_1-adrenoceptors on bovine cultured adrenal ZFR cells, for two reasons, as discussed below.

Firstly, the effects of several selective β-adrenoceptor agonists on hydrocortisone secretion were consistent with this classification. Isoprenaline, noradrenaline and dobutamine were all full agonists, whereas salbutamol acted as a partial agonist, producing 70% of the maximum response. Salbutamol is known to act as a full agonist at β_2-adrenoceptors, but only as a partial agonist at β_1-adrenoceptors (Farmer *et al.*, 1970). This, in itself, suggests that the adrenergic stimulation of hydrocortisone secretion is mediated by β_1-adrenoceptors. Although BRL37344 produced stimulation of the cells at 10^{-5}M, it was the least potent of all the agonists studied and only produced 19% of the maximum response seen with isoprenaline. Previous studies have shown that, in systems thought to contain β_3-adrenoceptors, BRL37344 was a more potent agonist than isoprenaline (Arch *et al.*, 1984; Bond & Clarke, 1988). Hence, it is likely that BRL37344 is producing a non-specific stimulation, and that β_3-adrenoceptors are not present on bovine cultured adrenal ZFR cells.

Secondly, determination of the pA_2 values for the β_1-antagonist practolol and the β_2-antagonist ICI118,551 provided definitive evidence for the presence of β_1-receptors (Table 1). The pA_2 value for practolol of 6.85 (6.67–7.06) agrees well with published values (Table 1). The gradient of the Schild regression was significantly less than 1, slope = 0.90 (0.84–0.96), suggesting deviation from an ideal competitive antagonist. Practolol and ICI118,551 showed no partial agonist activity (results not shown), even though practolol is known to be a partial agonist in other systems (Kenakin & Black, 1978). It is possible that isoprenaline potentiated the partial agonist properties of practolol, leading to production of more hydrocortisone than expected and hence giving a Schild regression < 1.

The pA_2 value for the action of ICI118,551 at β_2-receptors has been found to be 9.26, and at β_1-receptors 7.17 (Bilski *et al.*, 1983). Hence, our experimental value of 7.14 (7.03–7.28) agrees well with the value for β_1-receptors. In this case, the gradient of the regression line of 0.99 (0.97–1.01) implies that ICI118,551 is acting as a pure competitive antagonist.

Although the occurrence of adrenergic control of steroidogenesis at the level of the adrenal cortex still remains to be established *in vivo*, an increasing number of observations suggest this possibility. Adrenergic innervation of the adrenal cortex has been demonstrated in man (Mikhail & Amin, 1969), the rat (Kleitman & Holzwarth, 1985) and the mouse (Migally, 1979). No similar studies on bovine adrenal cortex have been published.

Shima *et al.* (1984) have shown, using binding of [^{3}H]-dihydro-alprenolol, that membranes prepared from both the capsulated (zona glomerulosa, ZG) and decapsulated (ZFR) regions of the rat adrenal cortex contain β_1-adrenoceptors, and that only the latter are coupled to adenylate cyclase *in vitro*.

Previous work on primary cultures of adrenocortical cells has shown a steroidogenic response to catecholamines in rat ZG cells (DeLean *et al.*, 1984), in a bovine mixed adrenocortical preparation (Kawamura *et al.*, 1984), and in bovine purified ZFR cells (Walker *et al.*, 1988).

In conclusion, we have shown that β_1-adrenoceptors are responsible for mediating catecholamine-stimulated hydrocortisone secretion from primary cultures of bovine adrenal ZFR cells. Ungar (1979) suggested that β_1-adrenoceptors tend to be associated with adrenergic nerve endings so that the occurrence *in vivo* of catecholamine-stimulated steroidogenesis is probably mediated by direct adrenergic innervation of adrenocortical cells.

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